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Functional expression of purinergic P2 receptors and transient receptor potential channels by the human urothelium

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Shabir S, Cross W, Kirkwood LA, Pearson JF, Appleby PA, Walker D, Eardley I, Southgate J. Functional expression of purinergic P2 receptors and transient receptor potential channels by the human urothelium. *Am J Physiol Renal Physiol* 305: F396–F406, 2013. First published May 29, 2013; doi:10.1152/ajprenal.00127.2013.—In addition to its role as a physical barrier, the urothelium is considered to play an active role in mechanosensation. A key mechanism is the release of transient mediators that activate purinergic P2 receptors and transient receptor potential (TRP) channels to effect changes in intracellular Ca^{2+}. Despite the implied importance of these receptors and channels in urothelial tissue homeostasis and dysfunctional bladder disease, little is known about their functional expression by the human urothelium. To evaluate the expression and function of P2X and P2Y receptors and TRP channels, the human ureter and bladder were used to separate urothelial and stromal tissues for RNA isolation and cell culture. RT-PCR using stringently designed primer sets was used to establish which P2 and TRP species were expressed at the transcript level, and selective agonists/antagonists were used to confirm functional expression by monitoring changes in intracellular Ca^{2+} and in a scratch repair assay. The results confirmed the functional expression of $P2Y_4$ receptors and excluded nonexpressed receptors/channels ($P2X_1$, $P2X_3$, $P2X_6$, $P2Y_6$, $P2Y_{11}$, TRPV5, and TRPM8), while a dearth of specific agonists confounded the functional validation of expressed $P2X_2$, $P2X_4$, $P2Y_1$, $P2Y_2$, TRPV2, TRPV3, TRPV6 and TRPM7 receptors/channels. Although a conventional response was elicited in control stromal-derived cells, the urothelial cell response to well-characterized TRPV1 and TRPV4 agonists/antagonists revealed unexpected anomalies. In addition, agonists that invoked an increase in intracellular Ca^{2+} promoted urothelial scratch repair, presumably through the release of ATP. The study raises important questions about the ligand selectivity of receptor/channel targets expressed by the urothelium. These pathways are important in urothelial tissue homeostasis, and this opens the possibility of selective drug targeting.

calcium; purinergic; transient receptor potential channel; urothelium

THERE HAS BEEN a growing appreciation that rather than a simple passive barrier, the urothelium plays a more active role in the urinary tract. After physical or other damage, the urothelium will self-repair by switching from a mitotically quiescent to a highly regenerative state (20, 28). The urothelium has inbuilt mechanisms for responding to changes in urinary salt concentration (18) and for initiating the innate immune system after exposure to uropathogenic *Escherichia coli* (21). Thus, the urothelium may be considered a hub for sensing and transducing information to other tissue compartments and acting to coordinate physiological bladder function and responses.

More intriguingly, the urothelium has been reported to possess sensory neuronal-like properties and to respond to mechanical and chemical stimulation through the release of transient mediators (4). Various mediators have been implicated, including ATP, nitric oxide, acetylcholine, and substance P (1, 7, 11). These short-lived mediators are considered to actuate suburothelial afferent neurons involved in the regulation of sensory perception and pain, but the urothelium is itself widely reported to express an array of receptors and channels that may respond in an autocrine/paracrine fashion to released mediators. These include purinergic P2X and P2Y (8, 24, 27), transient receptor potential (TRPV1, TRPV2, TRPV4, and TRPM8), acetylcholine (nicotinic and muscarinic), tachykinin, nerve growth factor, endothelin, sphingosine-1-phosphate, and bradykinin (3, 9, 15, 17) receptors. The outcome of such signaling is incompletely understood as it may play a bidirectional feedback role in modulating the neuronal signal and/or effect changes in urothelial homeostasis, such as barrier repair. It has also been suggested that abnormal expression of receptors and/or mediator release by the urothelium may be involved in dysfunctional diseases of the bladder, including idiopathic detrusor instability and interstitial cystitis (5, 25, 27).

Despite the literature reporting expression of these channels and receptors by the urothelium, consensus is confounded by contradictions in experimental approaches, including the species, specificity of reagents, and the nature of the tissue preparation (for a review, see Ref. 30). There has been limited characterization of these receptor/mediator signaling pathways in the human urothelium, where functional TRPV1 (10) and an autocrine-activated P2Y receptor pathway (19, 26) have been reported. Ultimately, this conflict and the lack of consensus are hindrances to the development of selective drugs.

To attribute expression and function to specific tissue compartments, the present study was designed to define the functional expression of purinergic and transient receptors in the isolated human urothelium and stromal cells in situ and in vitro. A preliminary investigation revealed a lack of specificity of commercially available antibodies. For this reason, our rationalized experimental approach was to identify candidate receptors based on mRNA expression followed by confirmatory functional experiments to measure changes in intracellular Ca^{2+} using specific agonists/antagonists. Finally, to examine whether receptor activation plays a role in urothelial homeostasis, we examined the effect of receptor activation on human urothelial scratch wound repair in vitro.

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Table 1. *RT-PCR primers*

Gene	Sequence	Product Size, bp
GAPDH		
Forward	5′-ACCCAGAAGACTGTGGATGG-3′	201
Reverse	5′-TTCTAGACGGCAGGTCAGG-3′	
$P2X_1$		
Forward	5′-GTGGAGAACGGGACCAACTA-3′	233
Reverse	5′-CCCATGTCCTCAGCGTATTT-3′	
$P2X_2$		
Forward	5′-TGTCATCGGGGTCATTATCA-3′	160
Reverse	5′-GGTGGTGCCATTGATCTTGT-3′	
$P2X_3$		
Forward	5′-CTTGCACGAGAAGGCTTACC-3′	199
Reverse	5′-CTCTGGGCAGAATCCTTGCA-3′	
$P2X_4$		
Forward	5′-ATCCAGGTCAACTGGGACTG-3′	112
Reverse	5′-AGCCAGGAGATACGTTGTGC-3′	
$P2X_5$		
Forward	5′-TGGGTGTTCCTGATAAAGAAGG-3′	103
Reverse	5′-GATCCGAGGTGTTGGTGAAG-3′	
$P2X_6$		
Forward	5′-GGCTCTCCTCGCCAAAAA-3′	105
Reverse	5′-CCAAGCTCCTTGATCTGAGTG-3′	
$P2X_7$		
Forward	5′-CGCCAAGTACTACAAGGAAAACA-3′	150
Reverse	5′-CCGAAGTAGGAGAGGGTTGA-3′	
$P2Y_1$		
Forward	5′-TTACTACCTGCCGGCTGTCT-3′	162
Reverse	5′-GGCAGAGTCAGCACGTACAA-3′	
$P2Y_2$		
Forward	5′-TCCTCTTCCTCACCTGCATC-3′	250
Reverse	5′-CCCAGCATGACTGAGCTGTA-3′	
$P2Y_4$		
Forward	5′-TGCCTGGTCACTCTTGTTTG-3′	205
Reverse	5′-GTACTCGGCAGTCAGCTTCC-3′	
$P2Y_6$		
Forward	5′-GCCACCCACTATATGCCCTA-3′	211
Reverse	5′-GAAAAGGCAGGAAGCTGATG-3′	
$P2Y_{11}$		
Forward	5′-CTGGTGGTTGAGTTCCTGGT-3′	234
Reverse	5′-GTTGCAGGTGAAGAGGAAGC-3′	
TRPV1		
Forward	5′-GCCTGAAGGAGCTTGTCAAC-3′	172
Reverse	5′-CGCCCTTTGGTTTTCTTAAA-3′	
TRPV2		
Forward	5′-CGCCATTGAGAAGAGGAGTC-3′	172
Reverse	5′-GCTTACCACATCCCACTGCT-3′	
TRPV3		
Forward	5′-GCGTGGAGGAGTTGGTAGAG-3′	239
Reverse	5′-CTCTGTGTACTCGGCGTTGA-3′	
TRPV4		
Forward	5′-ATCGTCTCAGCAGCCCTCTA-3′	168
Reverse	5′-TCGGAAAAGGTCCTTGAAGA-3′	
TRPV5		
Forward	5′-CACCACATGTGAGGCTTTTG-3′	153
Reverse	5′-ATGAGGTTGCGGGGACTAC-3′	
TRPV6 A		
Forward	5′-CTCGCTTCTTTGGACAGACC-3′	192
Reverse	5′-GGCCTAGCATCTGGAATCCT-3′	
TRPV6 B		
Forward	5′-GAGCCATGGGGGAAACAG-3′	153
Reverse	5′-TCACAACAGCGATGTGCAGT-3′	
TRPM7 A		
Forward	5′-TGGGAGGAACCTACAGATGC-3′	150
Reverse	5′-CTGCCCTATTGCCAAAAGAT-3′	
TRPM7 B		
Forward	5′-AACAGAGAGCAGCACAAAAGC-3′	231
Reverse	5′-AGTGGCTAAAGGCTAGCATGA-3′	
TRPM8 A		
Forward	5′-AATTTCCTACCGACGCCTTT-3′	220
Reverse	5′-TAGATGAGCCGGCTGAAGAT-3′	
TRPM8 B		
Forward	5′-GACGAGTCATTTTCTGTCTGGA-3′	226
Reverse	5′-ACCGAACGGAATATCCACCT-3′	

Sequences of human P2X, P2X, transient receptor potential (TRP)V, and TRPM primers used in the study designed from National Center for Biotechnology Information and Ensembl databases. GAPDH was included as a transcript control.

MATERIALS AND METHODS

Tissues and cell culture. Samples of the histologically normal ureter and bladder were obtained at surgery from adult patients (median age: 65 yr, range: 32–77 yr) with no history of urothelial dysplasia or neoplasia. The collection of surgical specimens had Research Ethics Committee approval and informed patient consent.

The urothelium was separated from the basement membrane with EDTA to recover sheets of the urothelium uncontaminated by stromal or neuronal cells (22, 23). The urothelial preparation was used to extract RNA (see below) or to establish finite normal human urothelial (NHU) cell lines, as detailed elsewhere (22, 23). NHU cells adopt a proliferative, nondifferentiated phenotype in serum-free culture, may be serially subcultured by trypsinization and, by modifying growth conditions, can be induced to undergo differentiation to a functional barrier urothelium.

An explant culture method was used to establish stromal cell cultures as previously described by Baker et al. (2), which comprised a mixture of smooth muscle cells and fibroblasts. Cultures were maintained in DMEM supplemented with 10% FBS (16).

RT-PCR. Total RNA was extracted from the freshly isolated urothelium or cultures using TRIzol (Invitrogen) and a DNA-free kit (Ambion). cDNA was synthesized using Superscript (Invitrogen). PCR was performed using Surestart *Taq* polymerase (Stratagene).

Primer pairs were designed and checked by basic local alignment search tool (BLAST) to detect specific P2X, P2Y, TRPV, and TRPM species without differentiating splice variants (Table 1). The optimal annealing temperature for each primer pair was determined by performing PCR with genomic DNA across an annealing temperature gradient from 50 to 60°C. PCRs were performed with an initial denaturation step of 95°C for 7 min, 30 cycles of 30 s at 95°C, 30 s at the optimal annealing temperature, extension for 30–100 s at 72°C (depending on the size of the expected product), and final extension at 72°C for 10 min. PCR products were separated on 2% agarose gels and visualized with Sybr Safe (Invitrogen). Controls comprised a reverse transcriptase-negative control for each RNA sample to eliminate DNA contamination and a no-template (water) control to monitor for PCR product contamination. Genomic DNA was used as the positive control for all primer sets.

Agonists and antagonists. Biochemicals were dissolved in the appropriate vehicle and diluted in HEPES-buffered HBSS (pH 7.4) supplemented, where required, by 2 mM Ca^{2+}. Agonists and antagonists were used at concentrations at or below the EC_{50} or IC_{50} as recommended by suppliers (Table 2). Vehicle controls were performed.

Intracellular Ca^{2+} measurements. For confocal imaging, proliferative urothelial cells derived from the ureter (unless otherwise stated) were grown on 0.1 mg/ml collagen-coated glass coverslips and loaded with 5 μM fluo4-AM and 5 μM fura red-AM in 0.02% pluronic acid in DMSO for 20 min (19). Coverslips were placed in a perfusion chamber (Warner Instruments) with a constant flow rate of 1.5 ml/min and positioned on a Revolution XD spinning disc confocal microscope (Andor) with a scan rate of 1 image/s.

For the fluorescent imaging plate reader (FLIPR), isolated ureter-derived NHU cells were seeded into tissue culture-treated black-

Table 2. *Specificity of agonists and inhibitors*

Agonist/Antagonist	Receptor (Order of Potency)	EC_{50}, μM	IC_{50}, μM	Supplier
ATP	$P2X_1$, $P2X_3$, $P2X_2$, $P2X_4$, $P2X_5$, $P2X_6$, $P2X_7$	Agonist		Sigma
	$P2Y_2$, $P2Y_4$, $P2Y_1$, $P2Y_{11}$			
UTP	$P2X_5$	Agonist		Sigma
	$P2Y_2$, $P2Y_4$, $P2Y_6$			
UDP	$P2Y_6$, $P2Y_4$, $P2Y_2$	Agonist		Sigma
ADP	$P2X_1$, $P2X_3$	Agonist		Sigma
	$P2Y_1$, $P2Y_{12}$, $P2Y_{13}$			
2-MeSADP	$P2X_4$	Agonist		Sigma
	$P2Y_1$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_6$	0.00009–0.019		
α,β-MeATP	$P2X_1$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$	Agonist		Sigma
BzATP	$P2X_1$, $P2X_4$, $P2X_2$, $P2X_5$, $P2X_7$, $P2X_3$	0.7		Tocris
	$P2Y_{11}$			
ARL 67156	Exto ATPase inhibitor		7.94–24	Tocris
TNP-ATP	$P2X_1$, $P2X_3$, $P2X_2$, $P2X_4$, $P2X_5$, $P2X_7$		0.001–0.006	Tocris
5-BDBD	$P2X_4$		0.5	Tocris
MRS-2365	$P2Y_1$	0.0004		Tocris
MRS-2768	$P2Y_2$	1.89		Tocris
UTPγS	$P2Y_2$, $P2Y_4$	10		Tocris
Capsaicin	TRPV1	0.08–10		Tocris
SB-366791	TRPV1		0.05–0.6	Tocris
GSK-1016790A	TRPV4	0.19–0.3		Sigma
RN-1734	TRPV4		2.3–10	Tocris
RN-1747	TRPV4	0.77		Tocris
PPADS	$P2X_1$, $P2X_2$, $P2X_3$, $P2X_5$, $P2X_6$, $P2X_7$		1–2.6	Sigma
	$P2Y_6$			
	$P2Y_2$		900	
	$P2Y_4$		1,500	

Data show the order of potency as well as EC_{50} and IC_{50} where available. A vehicle control (0.1% DMSO) was used where appropriate.

walled 96-well polystyrene plates (Costar) at 2×10^4 cells/well. After 24 h, the growth medium was replaced with loading medium (PBS containing 0.5 mM probenecid, 0.1% BSA, and 5 mM fluo4-AM) and incubated for 20 min at 37°C. Changes in cellular fluorescence were recorded using the FLIPR (Molecular Devices) after the addition of control buffer, ionomycin, or test compound.

Scratch repair assay. Cells were seeded into 24-well Primaria plates at 2.5×10^5 cells/well and cultured for 3 days before medium change and preincubation with drug/vehicle for 24 h. Cultures were scratched to create a 500-μm wound, rinsed to remove debris, and replaced with fresh medium containing the appropriate drugs. Digital images were acquired every 10 min over a 24-h period. The original wound area and the wound area remaining at the end of the period were measured by drawing a region of interest around the denuded space using Cell ^M software (Olympus), and the extent of healing, defined as the percent difference between the remaining versus original wound area, was evaluated.

Statistics. Statistics on scratch healing rates were carried out using one-way ANOVA with Tukey's multiple-comparison test using Graphpad Prism version 5 software (Graphpad Software).

RESULTS

Receptor transcript expression by freshly isolated and cultured human urothelium. Urothelium isolated from the bladder and ureter showed predominant expression of $P2X_4$ and $P2Y_1$ transcripts, with the bladder urothelium additionally expressing $P2Y_2$ (Fig. 1*A*). This profile was retained by urothelial cells in culture, with $P2X_2$ (bladder only), $P2X_5$, $P2X_7$ (ureter only), and $P2Y_4$ transcripts also detected in cultured cells. No urothelial expression of $P2X_1$, $P2X_3$, $P2X_6$, or $P2Y_6$ was detected, and expression of $P2Y_{11}$ was marginal. Stromal cell cultures expressed transcripts for $P2X_1$, $P2X_4$, $P2X_5$, $P2X_6$, $P2X_7$, $P2Y_1$, and $P2Y_4$ but not $P2X_2$, $P2X_3$, $P2Y_2$, $P2Y_6$, or $P2Y_{11}$.

Of the TRP channels, TRPM7 was expressed ubiquitously, alongside more variable expression of TRPV1, TRPV2, TRPV3, TRPV4, and TRPV6 transcripts (Fig. 1*B*). TRPV1 and TRPV2 were more readily detected in the bladder than in the ureter, and TRPV6 expression was differentiation associated, being expressed by the freshly isolated urothelium and by differentiated urothelial cell cultures. TRPM8 was expressed by the bladder only, whereas TRPV5 transcripts were completely absent. Stromal cells expressed TRPV1, TRPV2, TRPV4, and TRPM7.

Functional activation of purinergic receptors. In the absence of extracellular Ca^{2+}, application of exogenous ATP led to a rapid increase in intracellular Ca^{2+} (Fig. 2). ATP at 20 μM (the lowest concentration that produced a robust response in all cells) induced a signal that was close in amplitude to the ionomycin-induced maximum. The response to ATP mainly involved metabotropic (P2Y) rather than ionotropic (P2X) receptors, as *1*) the ATP response occurred in the absence of extracellular Ca^{2+}, demonstrating that the Ca^{2+} was released from the endoplasmic reticulum and was not dependent upon Ca^{2+} influx, and *2*) the ATP signal was largely attenuated by 100 μM PPADS, an inhibitor of purinergic receptors, which functioned here as a P2Y antagonist in the absence of extracellular Ca^{2+}.

In the presence of extracellular Ca^{2+}, selective agonists against $P2Y_2$ (MRS-2768) and $P2Y_4$ (UTPγS) each produced robust Ca^{2+} responses, but a $P2Y_1$ agonist (MRS-2365) failed to produce any response (Fig. 3). The lack of any selective $P2Y_2$ or $P2Y_4$ antagonists prevented us from inhibiting this Ca^{2+} response. In addition, as these experiments were carried out on proliferative urothelial cells derived from the ureter, which did not express $P2Y_2$ but gave a positive Ca^{2+} response,

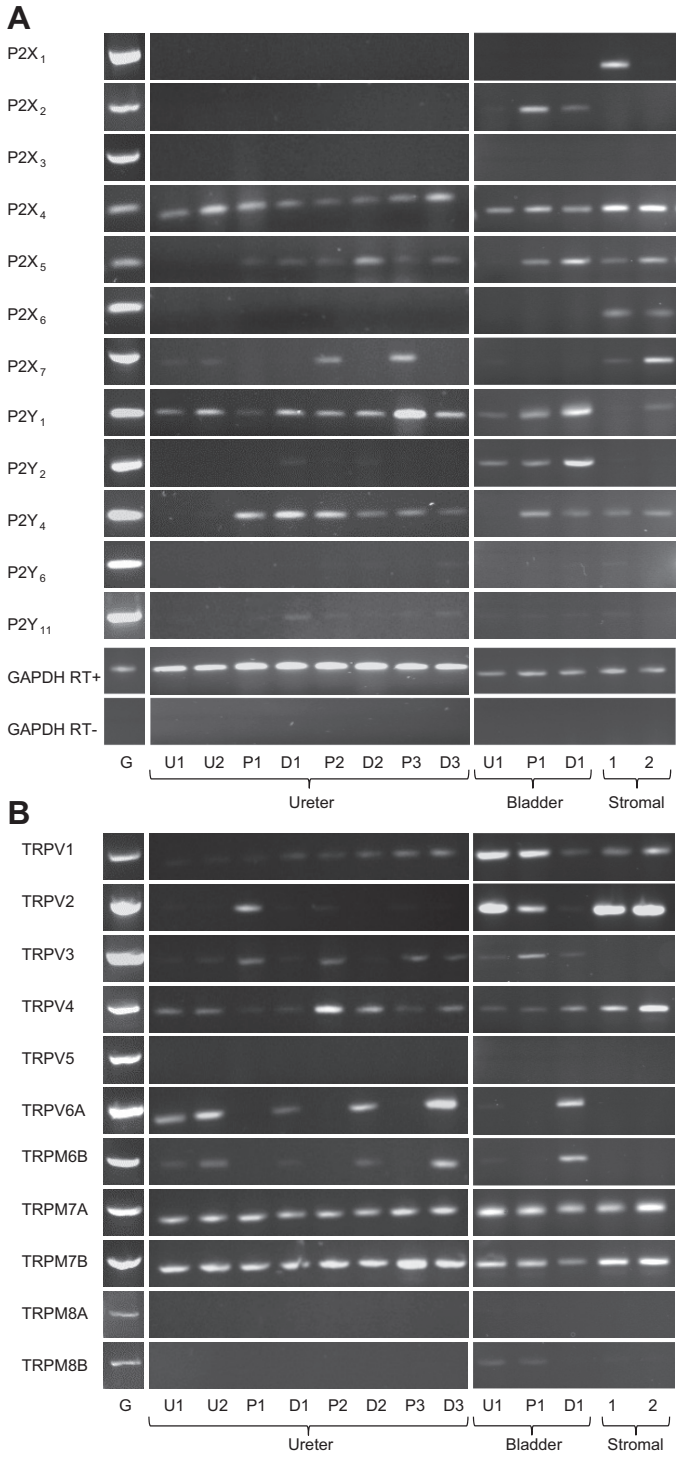


Fig. 1. Transcript analysis of purinergic (*A*) and transient receptor potential (TRP; *B*) channel mRNA expression by the urothelium. RT-PCR was carried out on freshly isolated urothelium from the ureter and bladder and on proliferative (P) and differentiated (D) normal human urothelial (NHU) cell cultures from three ureters and one bladder. For differentiation experiments, cultures were preconditioned for 4 days in 5% adult bovine serum (ABS) before being harvested and seeded into new culture flasks. After 24 h, exogenous Ca^{2+} was increased from 0.09 to 2 mM, and cultures were maintained in Keratinocyte Serum-Free medium (complete) supplemented with 5% ABS and 2 mM Ca^{2+} for a further 5 days before use. Stromal cultures from two independent donors were included as a positive control to examine the potential contribution of the nonurothelial compartment to receptor expression. RT, reverse transcriptase.

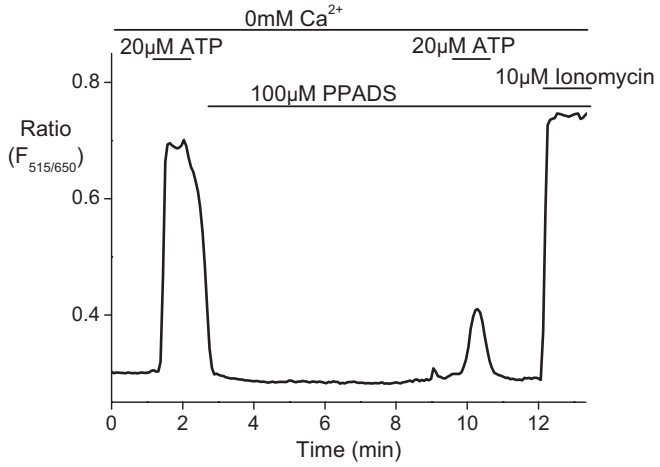


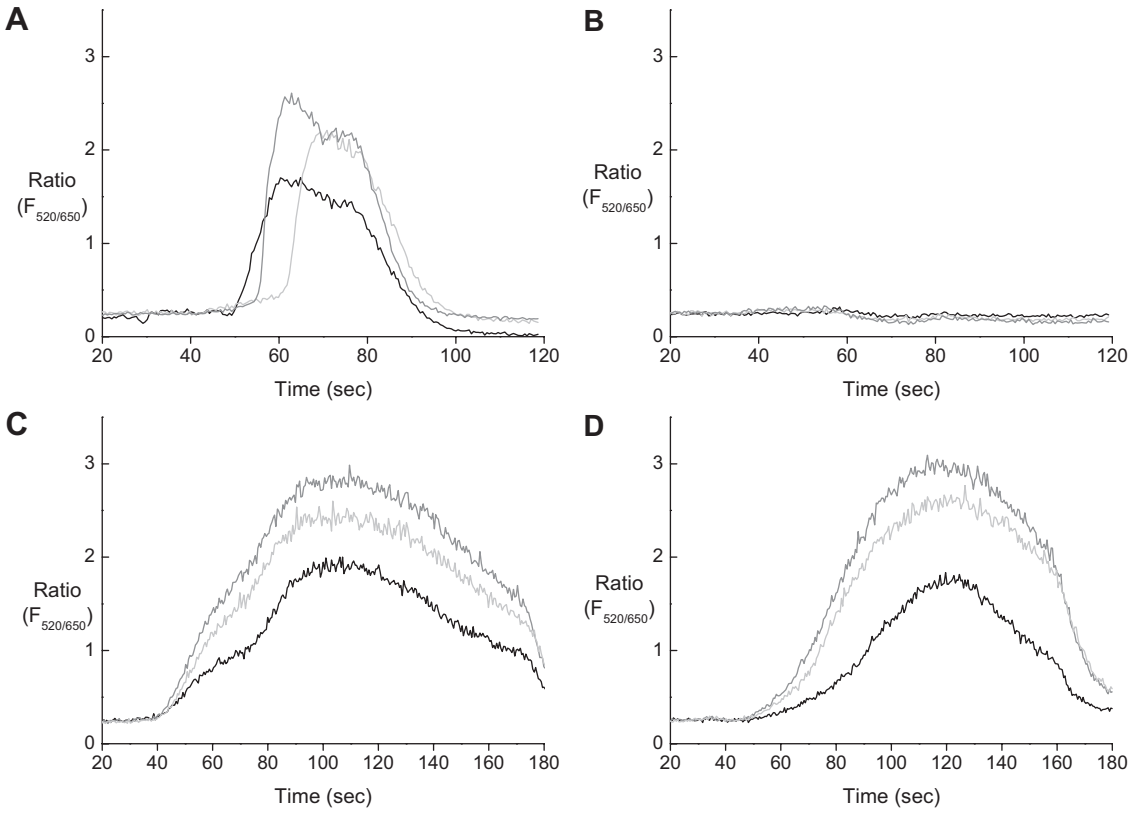
Fig. 2. Functional activation of P2 receptors. In the absence of extracellular Ca^{2+}, 20 μM ATP elicited an increase in intracellular Ca^{2+} that was partially inhibited by the addition of 100 μM PPADS. Ionomycin (10 μM) was used to elicit a maximum response.

there was little value in using MRS-2768 on differentiated cells that did express $P2Y_2$. Selective P2X antagonists were used to define the contribution of specific receptors to the ATP control response: TNP-ATP ($P2X_1$, $P2X_2$, and $P2X_3$ inhibitor) and 5-BDBD ($P2X_4$ inhibitor) had only minor effects on the amplitude of the control ATP response (Fig. 4).

To further characterize the specificity of the urothelial response, replicate cultures from one NHU cell line were exposed to a panel of P2 agonists over a concentration range, and responses were assessed by monitoring changes in intracellular Ca^{2+} by FLIPR (Fig. 5). The results were used to derive the EC_{50} for each agonist and a rank order of potency, with UTP (95% confidence interval: 0.53, 0.47–0.60 μM) > ATP (95% confidence interval: 0.67, 0.57–0.79 μM) > UTPγS (95% confidence interval: 2.02, 1.94–2.87 μM) > BzATP (95% confidence interval: 14.6, 11.8–18.5 μM) > UDP (95% confidence interval: 40.9, 36.8–45.5 μM) > ADP (95% confidence interval: 44.1, 38.8–50.1 μM). Two agonists, 2-MeSADP ($P2Y_1$, $P2Y_6$, $P2Y_{12}$, and $P2Y_{13}$) and α,β-MeATP ($P2X_1$ and $P2X_3$) did not elicit any response.

Functional activation of TRP receptors. The TRPV1 agonist capsaicin (1–100 μM) failed to elicit a Ca^{2+} transient in either proliferative (Fig. 6*A*) or differentiated (Fig. 6*B*) cultures from the ureter. Similarly, in the bladder, increasing (Fig. 6*C*) or decreasing (not shown) concentrations of capsaicin failed to produce Ca^{2+} transients in the majority of cells. A small subpopulation of cells (8 of 115 cells; Fig. 6*D*) showed a response, but only at toxic capsaicin concentrations (10–100 μM). In contrast, 1 μM capsaicin was able to elicit a consistent Ca^{2+} response in stromal cells (Fig. 6*E*), and this could be antagonized with 0.5 μM SB-366791 (TRPV1 inhibitor; Fig. 6*F*).

The TRPV4 agonist GSK-1016790A (0.1–1 μM) produced a Ca^{2+} response in both proliferative (Fig. 7*A*) and differentiated (Fig. 7*B*) NHU cell cultures that was robust and consistent. However, the Ca^{2+} response induced by GSK-1016790A was not attenuated by the TRPV4 inhibitor RN-1734 (Fig. 7*C*). To determine the specificity of GSK-1016790A, an alternative TRPV4 agonist, RN-1747, was used, but this failed to elicit any signal in NHU cell cultures (data not shown). In stromal



E

	Ureter			Bladder			Stromal
	U	Prolif	Diff	U	Prolif	Diff	Normal
$P2Y_1$	+++	+++	+++	+++	+++	+++	++
$P2Y_2$			+	+++	+++	+++	
$P2Y_4$		+++	+++		+++	+++	+++
$P2Y_6$							
$P2Y_{11}$							

Fig. 3. Functional activation of P2Y receptors. In proliferative, ureter-derived NHU cells, 25 μM ATP produced a strong response (*A*), whereas 1 nM MRS-2365, a $P2Y_1$ agonist, did not elicit any (*B*). Both 4 μM MRS-2768, a $P2Y_2$ agonist (*C*), and 20 μM UTPγS, a $P2Y_4$ agonist (*D*), produced robust Ca^{2+} responses. Graphs show the Ca^{2+} response of three randomly chosen representative cells. F520/650, fluorescence ratio at 520- to 650-nm wavelength. *E*: table summarizing PCR data, where the amount of product was qualitatively ranked as strong (+++), moderate (++), weak (+), or not expressed (blank).

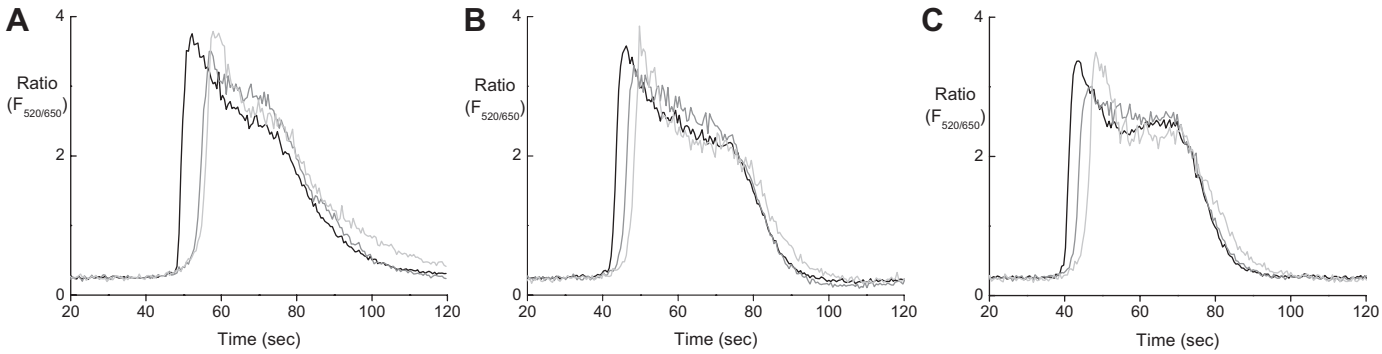
cells, RN-1747 produced a Ca^{2+} transient that was inhibited by RN-1734 (Fig. 7, *D* and *E*). Furthermore, in stromal cells, the Ca^{2+} transient induced by 0.1 μM GSK-1016790A could be inhibited by RN-1734 (Fig. 7, *F* and *G*).

Scratch healing experiments. The application of 20 μM ATP to scratches increased the healing rate in both proliferative and differentiated cell cultures, which could be reversed using the purinergic antagonist PPADS (Fig. 8). Neither capsaicin (TRPV1 agonist) nor SB-366791 (TRPV1 antagonist) had any significant effect on the rate of scratch repair in either proliferative or differentiated cell cultures (data not shown). GSK-1016790A increased the rate of scratch repair compared with untreated cells in a dose-dependent manner, with 1 μM having a significant effect in both proliferative and differentiated cell cultures. However, the TRPV4 antagonist RN-1734 had no effect on scratch repair itself and did not inhibit the increase induced by GSK-1016790A (Fig. 9).

DISCUSSION

As two major classes of receptors that can trigger changes in intracellular Ca^{2+}, the expression of purinergic P2 and TRP receptors by the urothelium is considered central to the pharmacophysiology of the bladder and urinary tract. However, as recently discussed (30), the literature lacks consensus. By adopting a systematic approach with stringent primer design, relevant positive controls, and cautious use of pharmacological agents, our aim was to develop a defined list of functional receptor expression by human urothelium. In Table 3, we summarize where the data is robust and where there remains discrepancy.

By combining ex vivo and in vitro approaches, we have shown that, at least at the transcript level, the expression of purinergic and TRP receptors is generally maintained in culture, although expression is modulated by differentiation sta-



D

	Ureter			Bladder			Stromal
	U	Prolif	Diff	U	Prolif	Diff	Normal
$P2X_1$							+++
$P2X_2$				+	+++	+++	
$P2X_3$							
$P2X_4$	+++	+++	+++	+++	+++	+++	+++
$P2X_5$		+++	+++		+++	+++	+++
$P2X_6$							+++
$P2X_7$	+	+++					+++

Fig. 4. Functional activation of P2X receptors. In proliferative, ureter-derived NHU cells, 25 μM ATP in control cells produced a strong response (*A*), whereas the addition of either 10 nM TNP-ATP, a $P2X_{1,2,3}$ inhibitor (*B*), or 1 μM 5-BDBD, a $P2X_4$ inhibitor (*C*), produced minimal inhibition of the ATP response. Graphs show the Ca^{2+} response of three randomly chosen representative cells. *D*: table summarizing PCR data, where the amount of product was qualitatively ranked as strong (+++), moderate (++), weak (+), or not expressed (blank).

tus. Interestingly, $P2Y_4$ transcripts were only expressed in vitro and not by the native urothelium. This finding highlights the dangers of drawing conclusions without the relevant experimental controls, but, although it may reflect an artifact of the culture conditions, it is worth considering whether this might represent an inducible urothelial response in some pathophysiological circumstances. We found some evidence for regional differences between bladder and ureteric urothelium, particularly relating to $P2Y_2$, $P2X_2$, and TRPM8 expression by the bladder. Such regional differences are perhaps not surprising given the differences in tissue function and embryological derivation of the bladder and ureter, although the full implications have yet to be determined. Here, we provide unequivocal evidence showing that the human urothelium expresses functional $P2Y_4$ receptors and that other receptors ($P2X_1$, $P2X_3$, $P2X_6$, $P2Y_6$, $P2Y_{11}$, TRPV5, and TRPM8) are not expressed, but, due to the inadequacy of specific reagents, other transcript-expressed P2X and P2Y receptors have yet to be confirmed functionally. Interestingly, urinary tract-derived stromal cells have been shown to express some receptors (e.g., $P2X_6$) that were not expressed by the urothelium at all. This is important and may go some way toward explaining some of the controversy in the experimental literature if there has been inadequate separation of urothelial from stromal cell types. In our culture approach, the urothelial compartment is first separated cleanly, at the level of the basement membrane, from the stroma and the urothelial cells are then cultured in a serum-free medium formulation that discourages stromal cell growth, resulting in a "pure" epithelial cell culture.

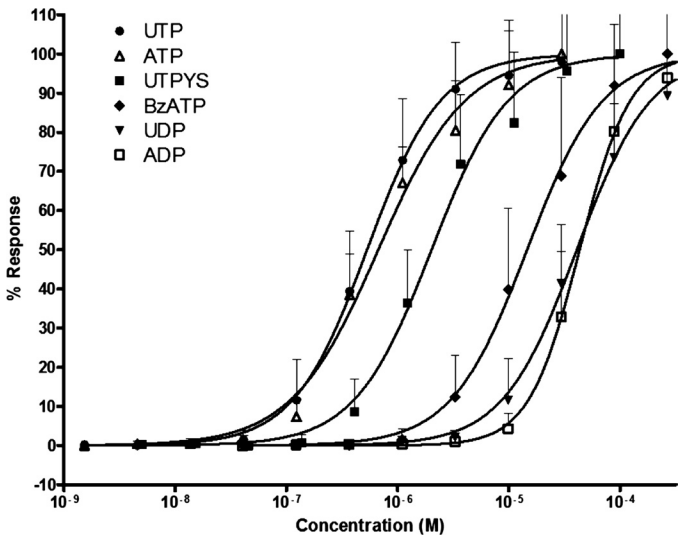


Fig. 5. Dose response of P2 agonists. Shown is the normalized dose response of P2 agonists added to NHU cultures to determine their potency. Fluorescent imaging plate reader (FLIPR) data represent an average of 8 independent wells.

A lack of specific agonists and antagonists for the TRP family of channels limited functional characterization, although expression of TRPV1, TRPV2, TRPV3, TRPV4, TRPV6, TRPM7, and TRPM8 transcripts was observed. One of our most intriguing findings involved TRPV4, where we found evidence of transcript expression but encountered reproducible discrepancies in functional activity. The well-characterized TRPV4 agonist RN-1747 produced a robust Ca^{2+} response in stromal cells that was inhibited by RN-1734, suggesting that the drugs were functioning correctly and specifically, but use of RN-1747 on NHU cells produced no Ca^{2+} response. An alternative agonist of TRPV4, GSK-1016790A, also produced a Ca^{2+} response in stromal cells, which was

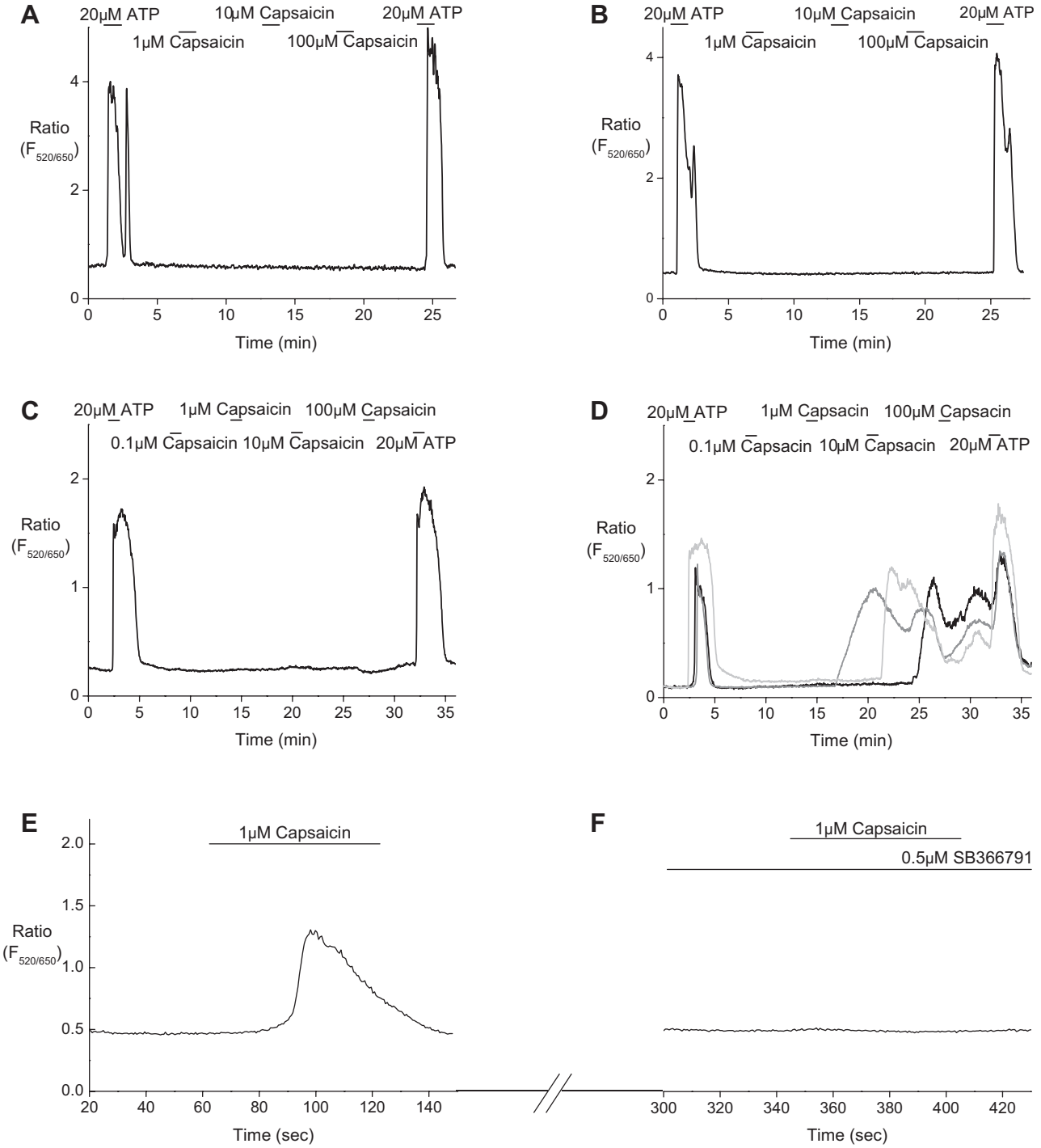
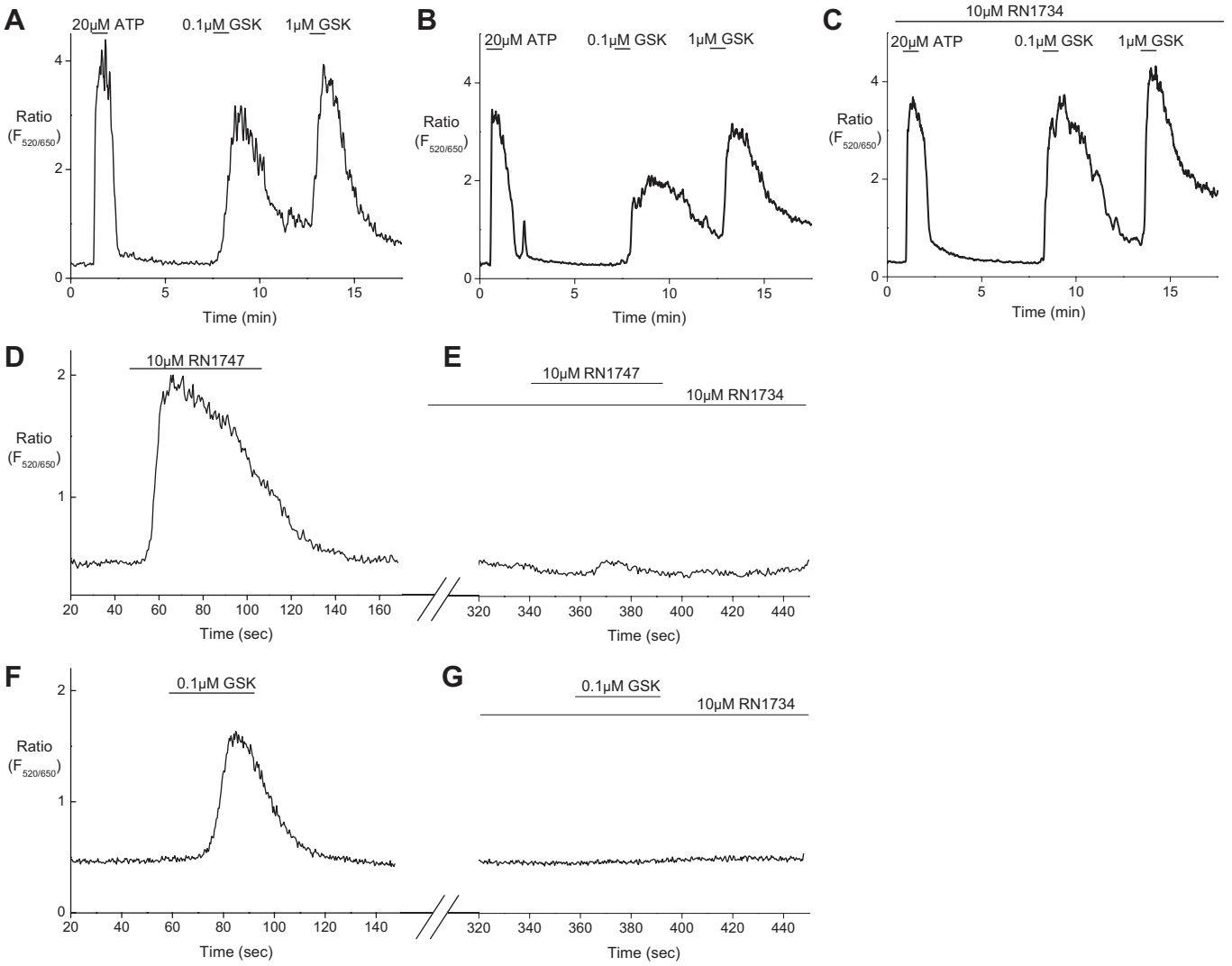


Fig. 6. Functional activation of TRPV1 receptors. After ATP application to ensure that cells were viable and responsive, concentrations of capsaicin up to 100 μM failed to elicit a Ca^{2+} response in either proliferative (*A*) or differentiated (*B*) cell cultures. Concentrations of capsaicin up to 100 μM also failed to elicit a Ca^{2+} response in bladder-derived cell cultures (*C*), although there was a very small subpopulation of cells that responded to higher concentrations of capsaicin (*D*). A concentration of 1 μM capsaicin was able to produce a Ca^{2+} response in stromal cells (*E*), which was inhibited by SB-366791 (*F*). Graphs show the mean Ca^{2+} response except for *D*, which shows Ca^{2+} responses from three individual cells.

inhibited by RN-1734. However, GSK-1016790A also produced a strong Ca^{2+} response in urothelial cells that could not be inhibited by RN-1734. The explanation for this inconsistency is currently unknown. The reagents used all invoked the expected responses in stromal cell cultures, suggesting that either GSK-1016790A has a selective off-target effect in the urothelium or that there is differential drug-binding selectivity of the TRPV4 receptor expressed by the human urothelium. Further work will be needed to determine if the differences seen in human urothelial cells are reflected in primary TRPV4 transcripts or in other (posttranslational) differences, but the results demonstrate the critical importance of incorporating



H

	Ureter			Bladder			Stromal
	U	Prolif	Diff	U	Prolif	Diff	Normal
TRPV1	+	+++	+++	+++	+++	+++	+++
TRPV2		+++	+	+++	+++	+	+++
TRPV3	++	+++	++	+++	+++	+++	
TRPV4	+++	+++	+++	+++	+++	+++	+++
TRPV5							
TRPV6	+++		+++	++		+++	
TRPM7							
TRPM8				++	++		

Fig. 7. Functional activation of TRPV4 receptors. A concentration of 0.1 μM GSK-1016790A (GSK) produced a Ca^{2+} response in both proliferative (*A*) and differentiated (*B*) cell cultures. The presence of the TRPV4 antagonist RN-1734 failed to inhibit GSK-induced Ca^{2+} responses in differentiated cultures (*C*) but could inhibit GSK-induced Ca^{2+} responses in stromal cells (*F* and *G*). The TRPV4 agonist RN-1747 produced a Ca^{2+} response in stromal cells (*D*), which could be inhibited by RN-1734 (*E*). *H*: table summarizing PCR data, where amount of product was qualitatively ranked as strong (+++), moderate (++), weak (+), or not expressed (blank).

appropriate controls even when using seemingly specific agonist and inhibitors. In contrast to our study, Everaerts et al. (12) reported TRPV4 expression in the mouse urothelium, which further highlights the problems of extrapolating results from animal models to humans.

Another area of controversy surrounds urothelial expression of TRPV1 (discussed in Ref. 31). Urothelial expression of TRPV1 has been reported in the mouse (30), rat (6), and human (10), but this has been contradicted by others (13, 14, 29). A major reason for this lack of consensus is the problem of poor

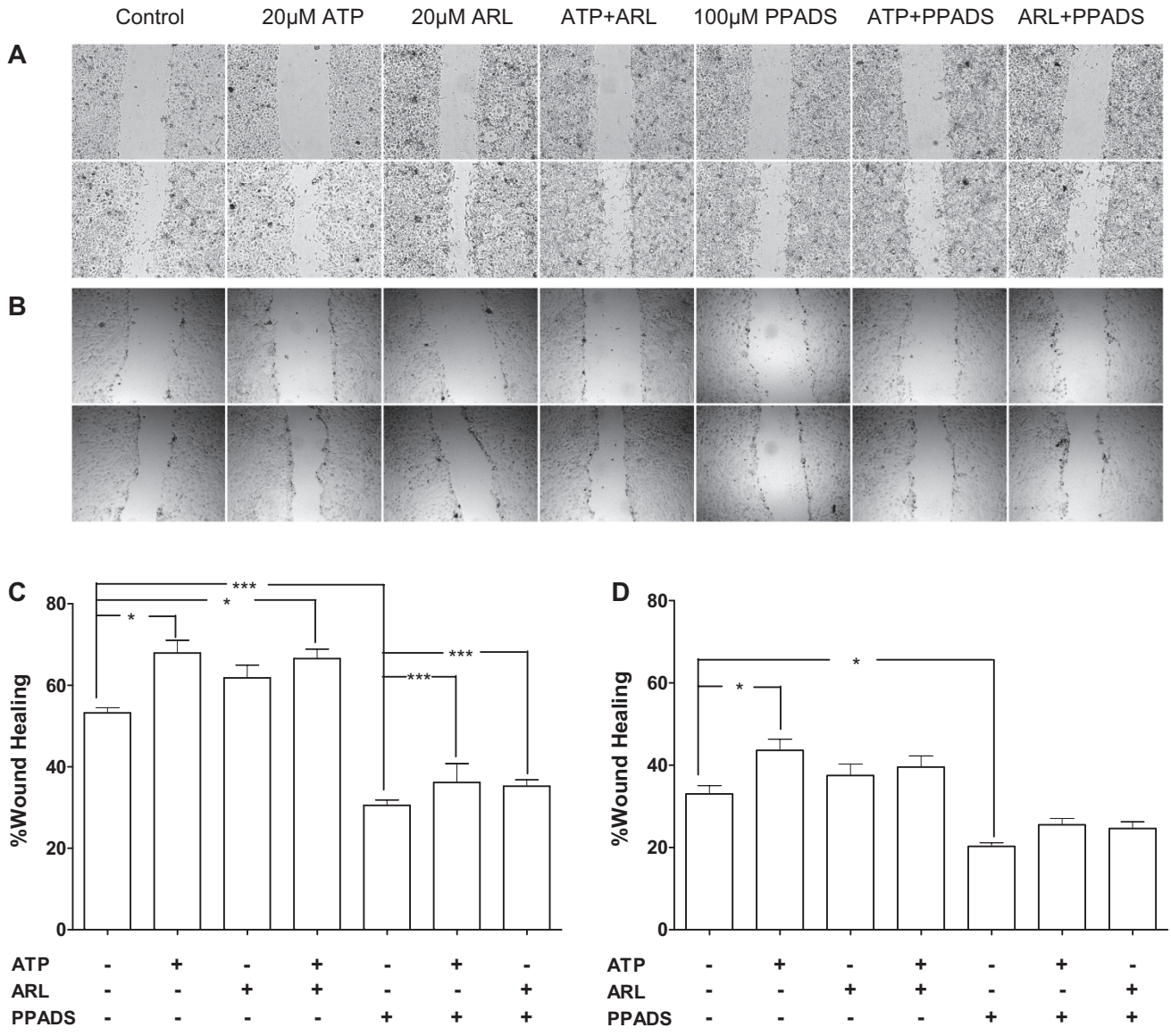


Fig. 8. Effect of purinergic signaling on scratch repair. *A* and *B*: images of scratch repair in proliferative (*A*) and differentiated (*B*) cell cultures. The addition of ATP and ARL-67156 (ARL) increased scratch repair in both proliferative (*C*) and differentiated (*D*) cell cultures, whereas the generic P2 inhibitor decreased scratch repair in both.

specificity antibody reagents, as exemplified by Everaerts et al. (13), who demonstrated that three independent TRPV1 antibodies gave indistinguishable immunoreactivity patterns on urothelia from wild-type TRPV1 and TRPV1$^{-/-}$ mice (13). As a background to our study, we tested a wide range of commercial antibodies against purinergic and TRP targets and found that none gave reliable results in human or rodent tissues, which is why we adopted an alternative characterization strategy.

We found that although TRPV1 transcripts were expressed by the urothelium, we failed to elicit any consistent response to capsaicin, despite invoking a robust response in stromal cells. In experiments where we directly measured intracellular Ca^{2+}, we found that the majority of NHU cells derived from the bladder failed to respond to capsaicin. In a very small number of cells, we detected a Ca^{2+} response to high concentrations of capsaicin that reflected a toxic effect. These results suggest that in the human urothelium, although TRPV1 is expressed at the mRNA level, there is no functional protein produced. We cannot explain the discrepancy between our results and those of another study (10) on cultured human urothelial cells, which reported a functional TRPV1 response to capsaicin and heat. The authors suggested that 1 μM capsaicin sensitized urothelial cells to further stimulation by capsaicin, results that we were unable to reproduce, and one possibility is that their cultures were contaminated by a subpopulation of stromal cells.

A motivation for this study was whether the presence and activation of P2 and TRP receptors plays a role in urothelial homeostasis, including regeneration. Here, we have shown that stimulation of P2 receptors with exogenous ATP enhanced scratch repair, as did the addition of the ecto-ATPase inhibitor ARL-67156, which prevents the breakdown of ATP. In contrast, blockade of P2 activity and Ca^{2+} signaling inhibited

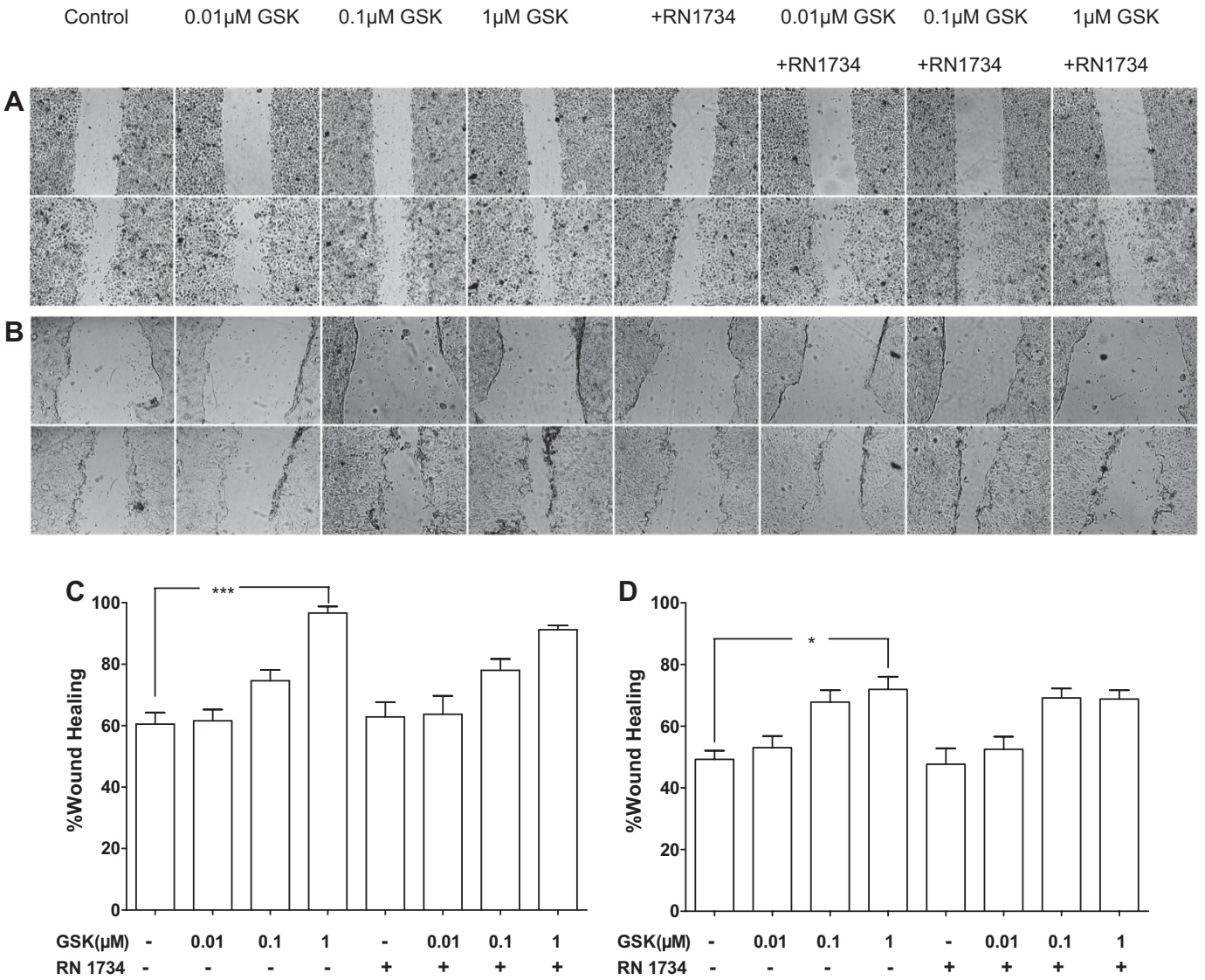


Fig. 9. Effect of TRPV4 agonists on scratch repair. *A* and *B*: images of scratch repair in proliferative (*A*) and differentiated (*B*) cell cultures. The addition of GSK increased scratch repair in both proliferative (*C*) and differentiated (*D*) cell cultures. The addition of RN-1734 has no effect.

Table 3. *Summary of P2 and TRP receptor expression*

P2	TRP
Positive Expression	
$P2X_2$*	TRPV2
$P2X_5$	TRPV3
$P2X_7$	TRPV6
$P2Y_4$†	TRPM7
No expression	
$P2X_1$†	TRPV5
$P2X_3$†	TRPM8
$P2X_6$	
$P2Y_6$	
$P2Y_{11}$	
Anomalous expression/function	
$P2X_4$	TRPV1
$P2Y_1$	TRPV4
$P2Y_2$	

*Bladder only. †Transcript expression result (positive or negative) was confirmed in functional experiments.

scratch repair in either the presence or absence of ATP (19, 26). This indicates that ATP is one of the major factors released upon damage and contributes to the scratch healing phenotype. We also found that GSK-1016790A increased the rate of scratch repair, but, as discussed above, until it is confirmed that in the urothelium this reagent acts through its reported TRPV4 target, its precise mode of action remains to be fully understood.

Conclusions. Our study contributes to understanding the expression and function of Ca^{2+}-modifying receptors and channels by the human urothelium and indicates the potential role that such receptors may play in urothelial repair and tissue homeostasis. The aim of our work was to tackle discrepancies in the literature, and although we would have liked to have produced definitive conclusions on what was expressed and functional by adopting a well-controlled analytical approach, we in fact identified new unknown inconsistencies in the specificities of apparently well-characterized agonists and antagonists. Our findings open new avenues for understanding

and exploiting drug selectivity for particular functional targets. As receptor subtype-specific ligands become available, this work can be carried forward to ascertain the role of these receptors in urothelial physiology and disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.S. and J.S. conception and design of research; S.S., W.C., and J.P. performed experiments; S.S., L.K., and J.S. analyzed data; S.S., L.K., and J.S. interpreted results of experiments; S.S. prepared figures; S.S., P.A., D.W., and J.S. drafted manuscript; S.S., P.A., D.W., I.E., and J.S. edited and revised manuscript, S.S., W.C., L.K., J.P., P.A., D.W., I.E., and J.S. approved final version of manuscript.

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